ANTIBIOSIS

by

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Thesis

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by

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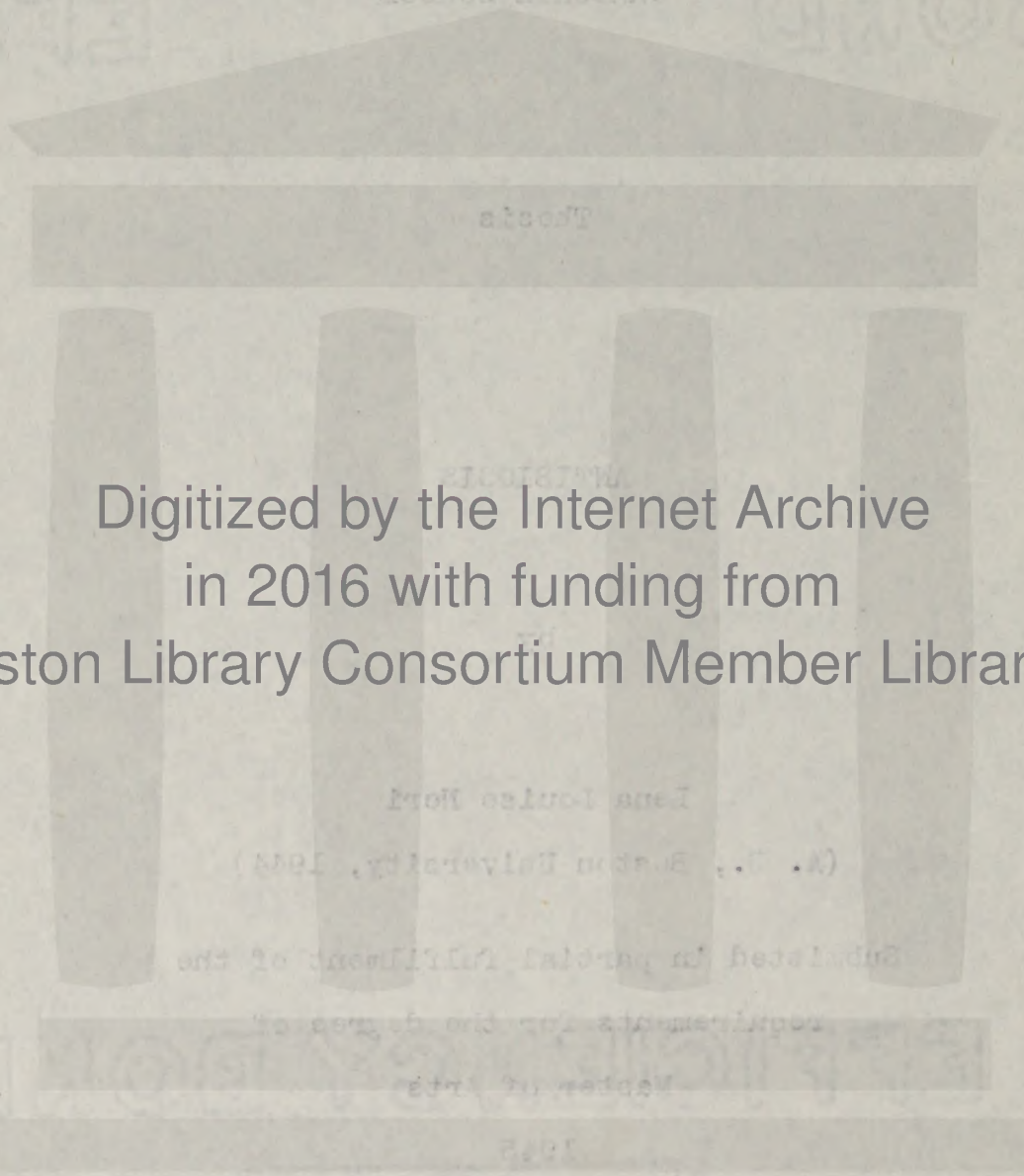

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INTRODUCTION

In recent years, the subject of bacterial association has received considerable attention. This increased interest is due largely to the greater attention which is being given to the finer metabolism of bacteria and the interactions which occur between the bacteria and their environment. Organisms are rarely, if ever, found growing as pure species in their natural habitat; mixed cultures of two or more species are the general rule. Since associations of bacteria are of such frequent occurrence, greater emphasis should be placed on studies of these relationships, in order to determine their effects on both the microorganisms and the environment.

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of one bacterium on another, but this is uncommon since one or the other usually dominates the picture. Associations may exist between different species of bacteria and bacteria with other classes of organisms, such as algae, protozoa, molds, and yeasts.

In many cases growth and multiplication are more vigorous in friendly associations than with either species existing alone. Such a phenomenon, where two or more organisms are living together in friendly association and are receiving mutual benefit, is referred to as symbiosis.

Certain soil bacteria belonging to the genus Rhizobium are found in tumors, or nodules produced on the roots of plants belonging chiefly to the family Leguminosae. These organisms utilize free atmospheric nitrogen and build it up into organic compounds. The plants are furnished available nitrogen by the synthetic activities of the organism, while the bacteria derive their other nutrients from the plant sap. The plant and bacteria live together for mutual benefit and a perfect symbiotic relationship exists.

Another favorable interrelationship, in which case only one member of the partnership is benefited, is called commensalism. The term "commensalism" may be defined as the living together of two species, one of which is benefited (nutritionally) by the association, while the other is apparently neither benefited nor harmed.

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Waksman and Lomanitz (1925) showed that Bacillus cereus developed very rapidly in a synthetic medium containing casein as the only source of nitrogen. The casein was vigorously hydrolyzed with the production of amino acids and other split products. The hydrolysis took place more rapidly in the absence of a fermentable carbohydrate. Pseudomonas fluorescens, on the other hand, was unable to attack and utilize the casein either for structure or for energy. However, this organism utilized amino acids with ease. B. cereus is a typical proteolytic organism capable of degrading native proteins to compounds having smaller molecular weights. P. fluorescens is nonproteolytic, but can utilize free amino acids as a source of nitrogen for structure and carbon for energy. When both organisms were inoculated into the above medium, the changes that took place in the casein molecule were different from those produced by each organism acting separately. B. cereus first attacked the casein with the liberation of amino acids and other compounds. P. fluorescens decomposed the amino acids as they were formed. The result was that the culture soon showed more cells of P. fluorescens than of B. cereus. P. fluorescens was definitely benefited by the association, while B. cereus was probably neither benefited nor harmed.

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Sears and Putnam (1923) were probably the first to conduct systematic studies of synergism. They reported that many pairs of organisms were observed to produce gas from sugar media, a reaction which was not produced by either organism in pure culture. They explained the phenomenon by stating that one of the organisms of the pair was capable of forming acid, while the other member produced the gas. The acid former degraded the carbohydrate and released a substance which was utilized by the second organism resulting in the production of gas. The substance attacked by the gas-forming member of the pair was not an end product of the action of the acid producer, but an intermediate product of metabolism.

Many organisms live or prey on the tissues of plants and animals; such an association is called parasitism. The parasite may, in rare cases, be nonpathogenic; that is, it may cause no damage to its host, as for example, Trypanosoma lewisi, which lives on rat tissue, and apparently excites no reaction there.

Most parasites, however, do some damage to their host and thus cause disease; this phenomenon is pathogenicity, and the disease producing agent is called a pathogen. Parasitism involves various degrees of pathogenicity, some microorganisms causing so much damage that they soon kill their host. The tubercle bacillus in man is an example of such a microorganism.

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A parasitic relationship may exist between lower forms. There are numerous fungi which spend essential parts of their life cycle inside the bodies of other fungi, deriving their food from them. Ampelomyces, parasitizing powdery mildew fungi, are common on species of the mold Saprolegnia. Frequently, zoospore formation of the host is suppressed and the parasite develops its sporangia and oöspores within hypertrophic galls of the host (Weindling, 1938). This is an example of obligate parasitism, since the microorganism cannot develop outside the host.

Between microorganisms, since the organisms do no live on each other, the injurious relationship is not parasitism, but "antibiosis". The term "antagonism" or "antibiosis" is employed chiefly for the situation where there is a clearly demonstrable harmful effect of one microorganism on another, or when a characteristic product fails to be formed or disappears in the mixed culture. It is probable that some metabolic waste product is produced which is without effect upon its producer but poisonous to its antagonist. This is well illustrated by the following example: meat, if left by itself, soon spoils, but if dropped into buttermilk will keep for some time. The lactic acid formed by the milk organisms is toxic to the putrefying bacteria, which normally decompose the meat.

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Associations of these various types determine to a great extent the conditions under which microorganisms can exist in nature. Antibiosis, in particular, plays a part in human affairs by causing the destruction of pathogenic bacteria in soil, water, and sewage, and thus cuts off these routes of transfer of infectious disease. In recent years, the isolation of specific antagonistic substances from microorganisms has made possible the inhibition or destruction of pathogens within the human body. Because of the special practical significance of this type of association, this paper will concern itself with the antagonistic relationships between microorganisms.

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It was as early as 1877 that Pasteur noted that the development of anthrax in sensitive animals can be repressed if the inoculation of Bacillus anthracis is accompanied by other bacteria, such as species of Streptococcus. Pasteur may be looked upon as the first to advance the idea of bacteriotherapy. It was soon established that when staphlylococci or anthrax, typhoid or diphtheria bacilli are added to the soil, they are rapidly antagonized by the soil microbes.

Some common saprophytes as Bacterium fluorescens and Bacterium pyocyaneum were found by Garré in 1887, Bouchard in 1889, and Freudenreich in 1888 to produce substances antagonistic to the colon-typhoid group of bacteria as well as to many others.

In 1904, Prescott and Baker observed that in a mixture of Escherichia coli and Streptococcus faecalis the former organism increased at a more rapid rate than the latter during the first few hours; then the S. faecalis gradually gained the ascendency and finally outgrew the E. coli present. In 1917, Savage and Wood worked with the same pair of organisms and made a similar observation.

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In 1918, Twort noticed certain transparent areas in a

culture of staphylococcus which were free from bacterial growth. He found that if he touched one of these areas with an inoculating loop and then streaked it over the surface of an agar culture of the same species of staphylococcus, clear transparent areas developed along the line of streaking. No contaminating organism was visible under the microscope. Twort also found that if the material from transparent areas was filtered through a Berkefeld filter, the filtrate contained a substance which was capable of dissolving a broth culture of the organisms; it was, therefore, a "filtrable virus". This lytic action was shown to be transmissable in series. D'Herelle, in 1916, independently of Twort, observed the same phenomenon and named the lytic principle bacteriophage. The term "bacteriophage", sometimes referred to simply as "phage", means literally "bacteria-eating agent".

Modern research has shown the close similarity of the bacteriophages to the recognized filtrable viruses. Like the viruses, the bacteriophages appear to be extremely minute, filtrable bodies, invisible by ordinary methods of microscopic examination, not cultivable on lifeless media, and increasing only in the presence of the particular living, growing bacterial cells which are susceptible to their action.

A number of bacteriophages, especially those active for the intestinal bacteria, have been obtained from feces, sewage, ground-up houseflies, etc. Extracts of chicken feces and horse

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manure have yielded phages specific for dysentery and typhoid organisms (D'Herelle, 1926).

A most interesting example of the inhibitory effect of bacteria in association was reported in 1920 by Theobald Smith and D. E. Smith. They found that Bacillus paratyphosus B, after it had grown in lactose bouillon for four to six days, prevented the development of gas by Bacillus coli when this was added. Members of the closely related hog cholera group had no such action in the given time; but after eighteen days growth, they also inhibited the gas production for the B. coli added at this time.

Greer and Nyhan, in 1928, prepared several pairs of organisms likely to be encountered in water supplies and observed the number of cells of each species at 24-hour intervals. Their results showed that one member of a pair almost invariably outgrew or destroyed the other member. In most cases the final result depended upon the proportions of each organism present at the beginning of the experiment. If one organism is present in greater numbers than the second organism, the former will tend to gain the ascendency; if the second organism is present in greater numbers than the first organism, the reverse will be true.

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The addition of gramicidin to nutrient broth prevented the growth of Gram-positive cocci, but was unable to retard the multiplication of any of the Gram-negative bacilli. Streptococci, incubated at 37° C. with gramicidin, lost the ability to reduce methylene blue, thus indicating an inactivation of the dehydrogenating enzyme of the organism. This occurred before

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In 1940, Hoogerheide and McDonald isolated a substance from soil bacilli that inhibited encapsulation of Friedländer's bacillus (Klebsiella pneumoniae)and was also highly bactericidal for Gram-positive microorganisms. The elaboration of a lytic principle was by no means restricted to one organism but was found in a number of species. The most common spore formers, such as Bacillus subtilis, Bacillus megatherium, Bacillus mesentericus, and B. cereus, excrete similar bactericidal products during growth in nutrient broth. A crystalline fraction obtained from the crude lytic material was found to be highly germicidal for many Gram-positive organisms when used in concentrations from 0.00001 to 0.02 mg per cubic centimeter of medium.

Waksman and Woodruff, in 1941, isolated an Actinomyces from soil which possessed strong bacteriostatic and bactericidal properties. The organism was found to belong to the chromogenic type of actinomycete, which produce a dark brown to black pigment on peptone or protein media. They named the organism Actinomyces antibioticus.

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The former fraction was found to be strongly bacteriostatic, whereas the latter was highly bactericidal. Actinomycin A was found to be bacteriostatic against all bacteria tested, although Gram-positive species were more sensitive to the compound than Gram-negative forms. Later in 1942, Waksman and Woodruff reported that actinomycin is a powerful bacteriostatic agent against Staphylococcus aureus, Clostridium perfringens (welchii), Streptococcus pyogenes, and Diplococcus pneumoniae Type 1. Over a 5-day period with or without the presence of ten percent serum, this bacteriostatic action slowly became bactericidal. The extract appeared to be more effective against pneumococci and streptococci than against staphylococci.

Many new antibiotic agents are being discovered. Dubos and Hotchkiss (1942) reported the antibiotic nature of tyrothricin, another substance obtained from autolysed cultures of the soil organism Bacillus brevis. It is composed of two substances, gramicidin, which is primarily bacteriostatic for some Gram-positive bacteria, and tyrocidine, which is bactericidal for some Gram-positive and a few Gram-negative bacteria. In the same year Waksman and Woodruff reported a new selective bacteriostatic and bactericidal agent, streptothricin, a substance produced from growing cultures of Actinomyces lavendulae. This is particularly effective against Gram-negative bacteria. Streptomycin, a compound obtained from the growth of Actinomyces griseus, is effective against both Gram-positive and Gram-

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ORGANISMS HAVING ANTIBIOTIC PROPERTIES

The organisms known at the present time capable of producing antibiotic substances may be classified into several broad groups: (1) molds, particularly the members of the genera Penicillium and Aspergillus, (2) various yeasts, (3) actinomycetes, especially those with aerial hyphae of the genus Streptomyces, (4) bacteria, both the spore-forming including Bacillus brevis, Bacillus mesentericus and Bacillus mycoides, and the non-spore-forming among which the pyocyaneus and fluorescens groups and members of the colon-typhoid group are most important, (5) viruses, and (6) lichens. The antagonistic capacity is widely distributed among microorganisms and is not limited to any one group of bacteria or fungi. Useful antibiotic agents have been obtained from representative types of molds, actinomycetes, spore-forming and non-spore-forming bacteria.

There is some evidence that there are antibiotics in higher plants. B. Tokin (1944) and his associates noted that a paste prepared from a small amount of macerated onion, garlic, or other allied plant immediately emits votatile substances which are lethal to yeast cultures of the Chabli or Nadsonia genera when placed at a distance of several centimeters for 1-5 minutes. Phytoncides was the name Tokin applied to these vola-

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tile substances. The action of phytoncides upon protozoa is extraordinary. None of the investigators was able to find a single species of protozoa that could withstand them. It has been determined that onion or garlic phytoncides kill staphylococci, streptococci, typhoid bacilli and a number of other bacteria.

MOLDS

The antagonistic effects of molds have received considerable attention since the antibacterial action of penicillin, a substance produced by various species of Penicillium, was first observed by Fleming in 1929. The fungus from which this substance was first produced was identified as Penicillium notatum, a blue-green mold characterized by the production of conidia from sterigmata, which are produced in clusters or whorls known as verticils. The spore heads are branched and they look somewhat like brushes.

In recent years Chain (1940) and Florey (1941) have reported extensively on the preparation and properties of this substance. Preparations which exert a marked antibacterial effect on a wide variety of bacteria have been made in the laboratory. A concentration of 0.01 to 0.1 gamma (1 gamma = $\frac{1}{1,000,000}$ gm) per cc is sufficient to inhibit the growth of 2,500,000 hemolyt-

ic streptococci. In certain instances the effect of penicillin seems to be bactericidal while in other cases it appears to be bacteriostatic (Hobby, Meyer, Dawson, and Chaffee, 1942). The active agent is a waste product of the growing mold. The penicillium may be grown in broth culture medium, such as Czapek-Dox broth containing 40 percent brown sugar and no glucose; in this medium the acidity first increases and then after five days, the medium becomes alkaline. The greatest penicillin concentration is found after ten days. Penicillin may be produced on a large scale by inoculating spore suspensions, previously prepared by cultivation of P. notatum on agar, into large flat bottles. The mold excretes three substances which are important: (1) the yellow pigment chrysogenin, which is removed by treatment with charcoal, (2) penicillin, the most active substance, (3) notatin, which is produced most readily when the acidity of the medium is great. These substances are excreted during seven to fourteen days of incubation. After incubation the fluid is clarified by centrifugation, and the penicillin is extracted and purified. One method of purifying is to acidify the broth and shake it with acetone. The acetone is separated and shaken with buffer solution; this is treated with ether and then the ether extract is passed through an adsorption column of $Al(OH)_3$ which adsorbs the penicillin in its upper portion. This yields its penicillin to ether again, and it is finally extracted with dilute NaOH.

Various methods of standardization of penicillin are used. The most satisfactory method is perhaps that described by Abraham and Chain (1941) and Foster and Woodruff (1943) known as the cylinder plate method or the cup assay. The principle of the assay is that of determining the inhibitory power of the unknown sample as compared with the inhibitory power of a standard penicillin preparation of known potency against the organism under identical conditions. Staphylococcus aureus (brought to this country from Oxford University) was first proposed by the Oxford group (Abraham et al, 1941) for use in the cup assay. Foster and Woodruff have considered a strain of Bacillus subtilis as more useful for this purpose. The cup assay method is as follows: a culture plate containing a medium is uniformly and heavily seeded with staphylococci or spores of B. subtilis. A small clear cylinder with a perfectly flat edge, made of porcelain or of a 12-mm length of glass tubing of 8-mm outside diameter, is sealed by touching it, while slightly heated, to the surface of the inoculated solid agar and leaving it there. Six such cylinders are the usual number placed in one plate. Into each white porcelain cylinder is placed a measured quantity of penicillin-containing extract. Following incubation under standard conditions, growth of bacterial colonies gives the medium a pebbled gray appearance, seen everywhere except around the cylinders; here growth has been inhibited by penicillin. The width of the zone of inhibition reveals the potency of the penicillin solution. Measurement of

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these zones of inhibition permits standardization of penicillin in Oxford units by comparison with zones produced under identical conditions of test by standard solutions of known unit strength.

The "Oxford unit" was devised by Florey at Oxford University. It is an arbitrary unit based on comparison with a stable solution held as a standard for reference at Oxford. The unit is that amount of penicillin which, in 1 cc of aqueous solution, gives the same degree of inhibition by the cup assay method as the original. This amount of penicillin is the least amount necessary to inhibit completely the growth of the standard test strain of S. aureus or B. subtilis in 50 cc of a standardized extract broth.

Penicillin has a strong antibacterial action. Gram-negative bacteria are least sensitive and pyogenic cocci most susceptible. It is soluble in alcohol, but insoluble in ether or chloroform; it is inactivated by oxidation and by evaporation at 40° C. to 45° C., in acid and alkaline solutions, although it is fairly stable at pH 5 to 6. Light, oxygen, hydrogen, and carbon dioxide either prevent its formation or bring about its rapid destruction (Waksman, 1937).

Five cultures of P. notatum produced penicillin in markedly varying degrees. Cultures derived from morphologically different colonies of the same strain also varied in their peni-

cillin production. McKee and Rake (1942) reported a tube dilution test which they used for determining the potency. They referred the results obtained to a stable calcium salt of penicillin having 30 Florey units per milligram, which was used as a control standard in all tests. Streptococcus pyogenes, S. aureus, Streptococcus viridans and Pneumococcus Types I, II, III were susceptible to the action of penicillin, while E. coli, the Friedländer bacillus, the Salmonellas, S. faecalis and Aerobacter aerogenes were not.

Toxicity test in mice show that purified penicillin is relatively non-toxic. A highly purified sodium salt of penicillin containing 240 Florey units per milligram, was at most only a quarter as toxic as a crude butanol extract containing only 15 Florey units per milligram (McKee and Rake, 1942).

Penicillin is effective against hemolytic streptococcus and pneumococcus infections, as well as various staphylococcal infections; it is used in controlling local lesions of the eye caused by S. aureus. It is effective against sulfonamide-resistant strains of pneumococci, although pneumococcus cultures can build up resistance against penicillin (Waksman, 1944).

Antibiotic substances have also been produced from representatives of the genus Aspergillus. These molds have a characteristic arrangement of the conidia and conidiophores. The unbranched conidiophore arises from an enlarged cell of the veg-

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etative mycelium, and terminates in a swollen portion which is called the vesicle. From the vesicle there are given off a number of little stalks or sterigmata, which in turn bear the chains of conidia. This arrangement presents the spores in a compact mass at the tip of the hypha (Henrici, 1944).

Aspergillus fumigatus, characterized by a large club-shaped vesicle, represents a type of antagonistic organism which produces several antibiotic substances. These differ in their chemical nature and in the range of their antibacterial action, or their antibiotic spectra. Of the three substances produced by this organism, namely fumigatin (another compound, spinulosin, is chemically related to fumigatin and is produced only by certain strains of this organism), fumigacin and gliotoxin, the first is the least active; fumigacin is more active, and gliotoxin is the most active (Waksman and Geiger, 1944). Gliotoxin also acts upon a greater number of bacteria than fumigacin, including various Gram-negative bacteria. Gliotoxin is more toxic to animals than fumigacin. Of these three compounds fumigacin, because of its lower toxicity to animals and its activity in vivo, offers the greatest promise as a chemotherapeutic agent. It is far less active, however, than penicillin.

One particular variant of a strain of Aspergillus flavus, a yellowish-green mold recognized by its spiny, septate conidiophores, has proven especially active in the production not only of aspergillic acid but also of a substance, closely resembling

penicillin, which is called flavicidin (McKee, Rake, and Houck, 1944). The ability of A. flavus to produce, under different methods of cultivation, two antibiotic substances of such diverse character as aspergillic acid and flavicidin suggested that a mutation might have occurred in the organism. The production of flavicidin instead of aspergillic acid by A. flavus appears to be a function both of type of medium and method of cultivation. Thus, when tryptone brown-sugar medium was used, aspergillic acid was produced in static cultures and flavicidin in shaken cultures, and method of cultivation was the determining factor. However, when a modified Czapek-Dox medium (page 21) in shallow layers was used flavicidin was produced. In this case the medium was unsatisfactory for aspergillic acid production but satisfactory for flavicidin.

White and Hill (1942) have reported that A. flavus shows activity only on tryptone or peptone media. The addition of about 0.5 percent glucose increases the titer slightly. From the filtrates they obtained a crystalline material, which contains the active antibacterial substance. This substance is soluble in ether, alcohol, acetone or acetic acid; it is insoluble in petroleum ether. It is insoluble in neutral water, but dissolves either in carbonates or in dilute acids. In a medium of 0.5 percent each of tryptone, glucose and sodium chloride it has shown inhibitory activity as high as 1:40,000 against group A beta-hemolytic streptococci and lower activity, in descending order, against S. aureus, S. faecalis, and E. coli.

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McKee and Houck (1943) have shown that the similarity in spectrum of activity of flavicidin and penicillin is very marked. As is well known penicillin, while very active against most Gram-positive organisms, has little activity against Gram-negative bacilli. This is likewise true of flavicidin. Other similarities of penicillin and flavicidin are that both protect mice in equal degree against pneumococcus infection; both are highly soluble, and hence are readily absorbed after parenteral inoculation and are quickly excreted by the kidneys; cultures resistant to the action of penicillin are resistant also to flavicidin but not to other antibiotic substances; and finally an enzyme active against penicillin is active also against flavicidin, but not against other antibiotic substances.

Flavicidin shows a somewhat greater toxicity than penicillin, which may be due to small amounts of contaminating aspergillic acid (McKee and Houck, 1943).

It frequently happens that when bacteria and molds are in a position to compete, the bacterial member is checked simultaneously with its fungus associate. At times the bacterial colony is more sharply arrested in its development than is the fungus. Reid (1934) studied the bacterial inhibitory properties of certain molds, finding that extracts from some of the molds investigated were able to inhibit the bacteria.

Aspergillus, Penicillium and Trichoderma which include

common molds of universal distribution and importance are considered to be saprophytic in habit. In the case of bacteria pathogenic to animals, a strongly saprophytic habit is generally associated with the ability to produce highly potent toxins. The most destructive types of these organisms are unable to establish balanced parasitic relationships. It is mot surprising, therefore, to find that saprophytic molds affecting other microorganisms act upon them primarily by means of powerful poisons (Weindling, 1938).

YEASTS

At various times evidence has been presented that yeasts contain substances toxic for themselves and for some bacteria and molds. There are also fractions from yeast which antagonize the toxic action of germicides for Aspergillus niger and Penicillium glabrum.

Rose yeasts (Torula suganii) were found to contain a substance which is antagonistic to molds but not to yeasts. The growth of A. niger was reduced by 60 percent to 70 percent and that of Aspergillus oryzae by 25 percent to 30 percent. The substance remained in the yeast cells. The active substance was soluble in acetone, alcohol, ether, and chloroform. Acetone-treated yeast had no antagonistic effect, but only a stimulating one (Waksman, 1945).

Cook and Kreke (1941) tested fractions of yeasts for their effect on the growth of E. coli and S. aureus. Complete killing of some of the organisms was obtained in about 2 percent or greater of the fraction; lower concentrations caused an increase in colony size.

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Rose yeasts (*Torula aurantii*) were found to contain a substance which is antagonistic to molds but not to yeasts. The growth of *A. niger* was reduced by 80 percent to 70 percent and that of *Aspergillus oryzae* by 25 percent to 30 percent. The substance remained in the yeast cells. The active substance was soluble in acetone, alcohol, ether, and chloroform. Acetone-treated yeast had no antagonistic effect, but only a stimulating one (Waksman, 1918).

Cook and Krebs (1941) tested fractions of yeasts for their effect on the growth of *E. coli* and *B. aurora*. Complete killing of some of the organisms was obtained in about 2 percent or greater of the fraction; lower concentrations caused an increase in colony size.

Yeasts were found to contain a substance which inhibits the action of sulfanilamide against *Streptococcus hemolyticus* and also other streptococci and pneumococci, but no relation-

ship between the growth-promoting properties and antisulfanilamide activity of the yeast extract could be established (Loomis, Hubbard, and Neter, 1941).

Of considerable interest is the fact that in the case of both molds and bacteria the type of growth is altered. For the molds grown on a modified Czapek's medium containing the depressant substance from yeast, this change consists of the production of thick gnarled hyphae, and the lack of conidia and pigment formation. The form of colony of E. coli on nutrient agar containing the depressant is changed from smooth to rough with increasing concentrations. Microscopically this change is associated with a great increase in the length of the E. coli cell and the development of tangled filamentous structures. When the cultures are returned to media free of the depressant, highly motile forms occur (Cook and Kreke, 1941). The relationship between the morphological and biochemical changes and the possible effect on virulence are being studied.

ACTINOMYCETES

The ability to antagonize various bacteria and molds is widely distributed among actinomycetes. They are mold-like fungi characterized by extremely fine hyphae which are normally non-septate and either lack nuclei or possess nuclei too minute to be seen under the microscope. They reproduce by conidia

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formed in chains at the tops of aerial hyphae, and sometimes also by a fragmentation of the hypha into segments much like the oïdia of higher fungi (Henrici, 1944).

Some of the most active antagonistic cultures have been studied in detail, and several antibiotic substances have been isolated. These antibiotic substances vary greatly in their chemical composition and in their mode of action. They are highly selective in nature, affecting different organisms in a different manner. They are not only bacteriostatic, but also bactericidal. Some are highly bacteriolytic, a property widely distributed among certain types of actinomycetes.

Gasperini (1890) is credited with being the first to recognize the ability of actinomycetes to destroy microbial cells. He observed that the filaments of Streptothrix foerstin may destroy the cell membrane of several bacteria and fungi. He reported that Streptothrix develops habitually in a spontaneous manner upon the surface of bacteria and other fungi, upon which it lives to a limited extent in the form of a parasite, due to the ability of its mycelium to digest the membrane of these lower fungi. Thirty years later, 1921, Lieske not only described the antagonistic effects of actinomycetes on S. aureus but also reported the dissolution of various dead or living bacteria, incorporated in water-agar, by actinomycetes streaked on the surface of this medium.

Actinomycetes possessing antagonistic properties against bacteria and fungi are widely distributed in nature, especially in soils and in composts. Although antagonistic forms were also found among the genera Proactinomyces and Micromonospora, they were most abundantly represented by menbers of the genus Actinomyces (Waksman et al, 1942).

The method of demonstrating actinomycetes and other antagonistic microorganisms in the soil, used by Waksman and Woodruff (1940) is as follows: agar is washed in distilled water and dissolved, so as to give 1.5 percent concentration. Two grams of K_2HPO_4 are added per liter; 10-milliliter portions of this agar are distributed in test tubes and sterilized. A washed suspension of the specific bacteria, obtained by cultivation on solid or in liquid nutrient media, is prepared and added to the washed agar, previously melted and placed in a water bath at 42° C. One-milliliter portions of the still viable bacterial suspension are added to the agar tubes. The bacteria are thoroughly mixed with the agar.

The soil to be tested for the presence and abundance of the antagonists is suspended in sterile tap water, using a series of dilutions from 1:10 to 1:10,000. One-milliliter portions of these dilutions are placed in sterile Petri dishes, and the bacterial agar prepared by the above procedure is added. The plates are well shaken, to distribute the soil suspension thoroughly, and incubated at 28° or 37° C. The presence of antago-

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nists can be demonstrated by the formation of clear zones surrounding the colonies of the latter after 1-10 days incubation of the plates. These colonies are then transferred to fresh bacterial agar plates and later isolated in pure cultures by the use of convenient media.

The type of activity exhibited by a species of Actinomyces belonging to the Actinomyces albus group has been extensively studied by Welsch (1942), who reported some interesting antibacterial properties of this organism. He found that this species, which he called Actinomyces G. (named thus in homage to Dr. A. Gratia, who was one of the first to undertake a systematic study of the occurrence of bacteriolytic organisms in nature) grows readily in liquid or agar mineral media containing heat-killed or chemically killed Gram-negative or Gram-positive bacteria, or living Gram-positive bacteria. The bacterial suspensions became clarified after a few hours in the case of heat-killed Gram-negative bacteria, and only after two or three days in the case of either heat-killed or living Gram-positive organisms. It is necessary that the bacterial suspensions be inoculated with a sporulating broth culture of Actinomyces G. and that a small amount of the culture medium be transferred together with the organism. The antagonist grows very scantily in suspensions of living Gram-negative bacteria where it produces no lysis at all. It dissolves killed bacteria suspended in nutrient broth, but does not affect liv-

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ing bacteria to any extent under these conditions. Welsch (1942) reported that sterile filtrates of broth-cultures of Actinomyces G. obtained after sporulation, and designated as "actinomycetin", dissolved suspensions of heat-killed Gram-negative bacteria in a few hours and in 24 hours those of heat-killed Gram-positive bacteria. They had no action, however, on most living Gram-positive bacteria, although partial lysis of Klebsiella pneumoniae, S. hemolyticus and S. aureus was obtained. They had no action on any Gram-negative organism.

Bacteriolysis of living bacteria by Actinomyces G. was visualized as a two-step reaction: first, the susceptible cells are killed by the selectively bactericidal lipoid; second, those dead cells are then dissolved by the bacteriolytic enzyme (which is responsible for the lysis of heat-killed bacteria). The phenomenon does not take place in complex culture-media, since the bactericidal action of the lipoid is greatly impaired under these conditions; and the presence of living Actinomyces is generally necessary, since free lipoid should be secreted in susceptible suspension (Welsch, 1942).

Waksman and Woodruff (1940) isolated from the soil a pigment-producing Actinomyces possessing strong antagonistic properties against bacteria and fungi. This organism was described as A. antibioticus (aerial mycelium on synthetic media is white), and the active principle, actinomycin, was isolated and purified.

It forms orange-red crystals, is soluble in ether, alcohol and other organic solvents, but not in petrol ether, and is sparingly soluble in water. This substance was not only highly bacteriostatic (especially to Gram-positive bacteria) and fungistatic, but was also extremely toxic to laboratory animals. Another active antagonistic organism was later isolated which was found to be a strain of A. lavendulae (aerial mycelium on synthetic media has a lavender shade). This organism produced a totally different type of antimicrobial substance, since it was much more active against Gram-negative bacteria than were other antibiotic substances of microbial origin. This substance was purified and concentrated, and designated as streptothricin (Waksman and Woodruff, 1942).

This active substance produced by actinomycetes is largely thermostable; it passes through a Seitz filter; it can be removed by charcoal and is, partly at least, ether soluble. The active substance was found to reduce, even in very low concentrations, the bacterial population of natural substrates, such as milk. When added to agar, it prevented the development of the great majority of soil bacteria and actinomycetes, but not of molds (Waksman and Woodruff, 1940).

Streptomycin, a substance obtained from the growth of Actinomyces griseus, has been found to be effective against Gram-positive organisms such as B. mycoides and Gram-negative

organisms such as P. fluorescens, P. aeruginosa, E. coli, Kleb. pneumoniae, Mycobacterium tuberculosis and a number of other organisms (Schatz and Waksman, 1944). It is a highly stable compound resistant to destruction by other organisms and it is comparatively non-toxic.

SPORE-FORMING BACTERIA

Bacteria, as agents possessing antibacterial properties, have received considerable attention ever since Pasteur (1877) discovered the antagonistic effects of bacteria against the anthrax organism.

The ability of spore-forming bacteria to produce bactericidal substances has been known for many years. As early as 1907, Nicolle reported that a culture of B. subtilis isolated from the air readily lysed pneumococci, typhoid, anthrax and other organisms in vitro. Other spore-forming bacteria such as Tyrothrix distortus, T. geniculatus and T. terius isolated from cheese by Duclaux, were shown by Nicolle to produce similar bactericidal substances. Pringsheim in 1920 isolated a spore-forming bacterium which appeared accidentally as a contaminant in plate cultures of Corynebacterium diphtheriae and formed a zone in which the latter failed to develop. The bacillus, considered to be of the Bacillus mesentericus type, also inhibited meningococci and other bacteria.

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Spore-forming bacteria are active antagonists against

diphtheria and pseudo-diphtheria organisms. Dubos (1939) isolated from a soil, enriched with various living bacteria, a Gram-negative organism (Bacillus brevis) which has a marked lytic effect against Gram-positive bacteria, including staphylococci, pneumococci, and others. The protein-free bactericidal material isolated from a spore bearing soil bacillus has been purified and three crystalline preparations highly bactericidal for Gram-positive microorganisms have been obtained (Hotchkiss and Dubos, 1940). Two acid substances are isolated by solution of the crude material in alcohol, precipitation with 15 volumes of ether and fractional crystallization of the dried precipitate from hot absolute alcohol. The third substance, which has been named gramicidin, is concentrated by repeatedly recovering the fraction which remains soluble in alcohol on the addition of 15 volumes of ether, but is insoluble in absolute ether. Crystallization is effected by extraction with a mixture of equal volumes of acetone and ether, evaporating the extracts, and cooling a solution of the residue in boiling acetone. From 100 gm of crude material, there were isolated in all about 60 gm of acid of which at least the larger part could be separated as one or the other of the two crystalline acids, and approximately 10 to 15 gm of crystalline gramicidin. It appears probable that other active substances may be present. Residual fractions of alcohol-insoluble material and ether-soluble matter (fatty acid) are inactive (Hotchkiss and Dubos, 1940).

Of the acids the one least soluble in alcohol, desig-

nated graminic acid, crystallizes as colorless hexagonal prisms or platelets. The melting point is 232° - 234° C. The other fraction, larger in amount and more soluble in slcohol, has been called gramidinic acid by Hotchkiss and Dubos (1940). It crystallizes in clusters of microscopic needles, melting with decomposition at about 230° C. Gramicidin crystallizes from acetone as characteristic spear-shaped colorless platelets. These show a melting point of 228° - 230° C.

All three substances are very effective in killing Gram-positive microorganisms. An actively growing Type I pneumococcus culture is sterilized within one hour by the addition of 10 micrograms of acids or by 5 micrograms of gramicidin (Hotchkiss and Dubos, 1940). Five, two or even one microgram of gramicidin when administered by the intraperitoneal route will protect a large percentage of mice infected intraperitoneally with 10,000 fatal doses of virulent Type I pneumococci. Graminic and gramidinic acids in even much larger quantities, on the other hand, will not prevent the death of the animal. Similar results have been obtained with other Gram-positive microorganisms, while Gram-negative organisms are unaffected either in vitro or in vivo (Hotchkiss and Dubos, 1940).

Gramicidin is regarded as highly toxic, 0.3 mg intraperitoneally killing mice and smaller quantities causing marked toxic reactions. The non-protective crystalline acids appear to be considerably less toxic.

Dubos and Hotchkiss (1942) revealed the fact that cultures of B. brevis yield two crystalline polypeptides, gramicidin and tyrocidine, which are both endowed with antibacterial activity but differ in many other biological and chemical properties. Tyrocidine inactivates completely the oxidation-reduction system of susceptible cells. In this respect tyrocidine behaves like common antiseptics, and perhaps especially like the cationic detergents, which it resembles in several respects. The other substance, gramicidin, causes, on the contrary, only limited injury to the bacterial cells and may either stimulate or depress some of their metabolic functions, according to the composition of the medium in which the test is carried out. In the presence of phosphate and potassium ions, for instance, there is observed a prolonged stimulation of metabolism, which is independent of the amount of gramicidin present. In the presence of ammonium ions, gramicidin causes a depression of oxygen uptake by staphylococci under conditions otherwise favorable for stimulation.

Whereas tyrocidine is toxic for all groups of bacteria, gramicidin is entirely inactive against Gram-negative bacilli. A number of strains of aerobic sporulating bacilli isolated from a variety of natural sources (soil, sewage, fermented cheese) have been found to exert a marked bactericidal effect against Gram-negative organisms. All of these sporulating bacilli produce substances related to, or identical with gram-

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icidin which is effective only against Gram-positive organisms. The same bacilli also yield other products which, under the proper experimental conditions, are equally bactericidal for both the Gram-positive and Gram-negative groups (Dubos and Hotchkiss, 1941).

Spore-forming bacteria are found to produce substances antagonistic not only to bacteria but also to molds. B. mesentericus grown on artificial media produces an active substance, which suppresses the growth of Helminthosporium sativum (Christensen and Davies, 1940). It increases sporulation, inhibits or retards spore germination, causes abnormal hyphal growth, and induces mutations in certain strains of the fungus. The substance is thermostable, diffusible, withstands freezing and desiccation, and does not deteriorate readily. It is destroyed by alkalis but not by acids, and it is inactivated or destroyed by certain molds and bacteria.

Hoogerheide (1940) also isolated from the soil an aerobic spore-forming bacillus which produces, when grown on liquid media, a very active substance capable of preventing Friedländer's bacillus, types A and B, from forming capsules.

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NON-SPORE-FORMING BACTERIA

Numerous non-spore-forming bacteria have been shown to antagonize other bacteria. Particular attention has been paid to the pyocyaneus and fluorescens groups and also to the members of the colon-typhoid group.

Pseudomonas aeruginosa has long been known to be antagonistic to other bacteria. This action has been ascribed to an enzyme called pyocyanase, the green pigment pyocyanin, and possibly a third substance.

Schoental (1941) has reinvestigated the chemical and biological nature of the antibacterial agents of P. aeruginosa cultures. She has isolated from such cultures three antibacterial substances: (1) pyocyanine, (2) a-oxphenazine, and (3) an almost colorless bacteriolytic substance. These substances are heat stable, resistant to pepsin, soluble in alcohol and can be extracted quantitatively by chloroform and dried by the lyophilic method. They are not enzymes.

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Tests of growth inhibition by a-oxyphenazine were made on 30 species of bacteria and inhibition failed to occur with seven only. The bacteriolytic substance (3) is strongly active against Vibrio comma, causing a lysis of the vibrios and gel formation. The three substances inhibit the respiration of Vibrio cholera and S. aureus.

The green pigment develops more readily in a shallow layer of 1 percent dextrose broth medium. Proper aeration appears to be the most important factor in the development of the toxic substance.

The toxin is not specific in its action but is more active against certain species than others. Molds are more resistant than bacteria. Spore-forming bacteria and micrococci are very sensitive while colon bacilli are resistant.

Frost established in 1904 that a number of different bacteria are able to exert a marked antagonism against Eberthella typhosa. P. fluorescens exhibited the strongest effect.

The activity of the influenza organism is largely dependent on the presence of accompanying bacteria (Wolf, 1920); some of these, particularly the micrococci, are favorable to its growth, whereas others such as P. aeruginosa and B. subtilis, are injurious. It was observed by Lewis (1929) that the growth of P. fluorescens in manured soil and in protein solution con-

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taining B. cereus is due to the antagonism of the former against the latter. P. fluorescens was found to produce a water soluble, thermostable substance which was toxic to various bacteria except the green fluorescent forms; it was also active against actinomycetes but not against molds.

It has been found that in mixed cultures, the colon organism gradually replaces the typhoid. Chatterjee (1909) and other investigators have noted that typhoid and paratyphoid bacteria fail to multiply when inoculated into a medium in which colon bacilli are previously grown. The antagonistic action of paratyphoid against typhoid bacteria has also been established. Fulton(1937) reported that when E. coli and Salmonella schottmulleri are grown in association, the second is at first inhibited, but after E. coli passes its maximun development, it also makes a good growth. The slow lactose-fermenting strains of E. coli which occurred in stools and also the inhibitory action found in certain stools seeded with E. typhosa was ascribed to the antagonistic action of the former. Young actively growing cultures of E. typhosa inhibit the growth of E. coli, while older cultures are non-antagonistic.

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ity of most of them by ordinary microscopes and their filterability through fine porcelain filters) have received little attention. It is known, however, that some microorganisms are capable of destroying viruses and that some viruses are capable of antagonizing other viruses.

It has been found that B. subtilis is capable of inactivating the virus of vesicular stomatitis and also staphylococcus phage. The culture filtrate of B. subtilis is also said to be capable of suppressing the activity of the virus of rabies when the two are injected into rabbits (Rakieten, M. L., Rakieten, T. L. and Doff, 1936).

Various microorganisms are capable of producing nontoxic substances which inactivate plant viruses. Fulton (1943) has shown that A. niger forms a substance capable of inactivating many different plant viruses; the effect of the inactivator was found to be exerted on the virus itself and not on the plant.

Many reports have been made concerning the interference of one virus by another. It has been shown that even an inactivated virus, whether a homologous or a heterologous strain, is capable of suppressing the development of the influenza virus (Henle, W., and Henle, G., 1943).

Delbrück and Luria (1942) studied in detail the ability of bacterial phages to interfere with the development of other phages. In order to gain some insight into the intracellular

processes of virus growth they tried the simultaneous action of two different viruses, α and γ upon the same host cell. They discovered a case of interference. Mixed infection of a bacterium with particles of both viruses results in complete suppression of the growth of one virus, while the other grows normally. The physical characteristics of the two viruses are different; the two viruses differ as much as any two viruses with a common host could possibly differ. The fact that the yield of virus per bacterium is nearly the same for both viruses, although the two viruses differ greatly in size, suggests that the number of particles synthesized is limited by the availability of some substrate, a definite amount of which enters into each virus particle, either of type α or of type γ. The saturation may be due to the fact that among the bacterial enzymes which are necessary for virus synthesis, there is one "key-enzyme" which is completely engaged by one virus particle. Other virus particles coming later either remain idle or displace the first one from the key-enzyme. Thus, in multiple infection, only one particle grows. Multiple infection of a bacterium with several particles of the same virus has qualitatively and quantitatively the same effects as infection with a single virus particle. Thus Delbrück and Luria (1943) formulated a theory of the growth mechanism of bacterial viruses. Virus is considered to be produced with the intervention of a "key-enzyme" present in limited amount in each bacterial cell. Later Delbrück and Luria (1943a) showed that a bacterial virus, γ, aft-

er inactivation by ultraviolet radiation, retains its ability to interfere with the growth of another virus, α, acting upon the same host. A single partially inactivated particle is sufficient to suppress the growth of virus α in one bacterium. The partially inactivated virus γ is adsorbed by the sensitive bacteria and it inhibits their growth without producing lysis. The partially inactivated virus γ interferes also with the growth of active virus γ. The interfering activity of virus γ, although more resistant to radiation than the reproducing activity, is progressively destroyed by larger doses of ultraviolet rays. These results seem to support the hypothesis that interference between bacterial viruses is due to competition for a key-enzyme present in limited amount in each bacterial cell. They suggest that this enzyme is also essential for the bacterial growth.

These results show the possibility of rendering bacterial cells insensitive to virus by treatment with the ultraviolet inactivated virus, and they suggest that in order to be used as vaccine, a virus should receive the minimum dose of radiation sufficient to destroy its infectivity, because the protecting activity is itself slowly destroyed by the radiation. Since partially inactivated virus can not reproduce itself, the vaccine should be used in amounts large enough to block most or all of the spots in which active virus could grow.

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LICHENS

Just recently, it has been shown that the phenomenon of antibiosis is well exemplified in the lichens. A lichen consists of a fungus, generally an ascomycete, and a green alga. Burkholder and Evans (1945) have made a study of approximately one hundred kinds of lichens leading them to data concerning the antagonistic effects. Extracts were prepared by grinding with a glass mortar and pestle 100 mg of lichen in 1cc of phosphate buffer solution adjusted to pH 7.4. The reaction of the aqueous suspensions of lichen materials was measured with color indicator papers before making the tests, and if necessary, the pH was adjusted with diluted alkali to 7.0. They used the common assay procedure, previously described (page 22) in order to test the antibiotic potency of the extracts against bacteria in Petri-dish cultures; 0.2 ml of each lichen preparation was transferred to a cup. After a period of incubation of about 15 hours at 37° C., zones of bacterial growth-inhibition around the cylinders were an indication of antibacterial potency; the results of their tests with different lichen extracts show varying degrees of activity ranging from strong growth promotion to marked inhibition. The extent to which diffusion of the antibiotic substance takes place prior to growth of the test bacteria is a

LICHENS

determining factor of the diameter of the zone of inhibition. Tests with Sarcina lutea made with lichen materials which had been allowed to dry in the laboratory for several months showed zones of inhibition about the cylinders; this indicated the relative stability of some of the antibiotic lichen compounds. The activity of several species of lichens was not lost by boiling in Na_2CO_3 solution for several minutes.

They found that generally samples of a species of lichen collected from different regions show characteristic activity in antibiotic tests with suitable bacteria. Variability was shown in some samples which had previously given indication of activity and failed to inhibit bacteria a second time. A possible explanation for this may be the varying amounts of acids in different samples of some species of lichens. The occurrence of various characteristic acids in species of Cladonia have been considered as a possible explanation of antibacterial activities. In the group of 35 active species of Cladonia, characteristic compounds were found with varying frequency in the different kinds of lichens. The structural similarity between known inhihitory substances and certain lichen acids seemed to indicate that antibiosis in lichens is related to the constituent diagnostic compounds. Of the approximately one hundred kinds of lichens tested fifty-two were found to inhibit either B. subtilis or S. aureus or both of these species. Gram-positive bacteria including several pathogenic types are inhibited; how-

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ever, Gram-negative bacteria are generally not susceptible to the antibiotic substance of lichens. Whether the active agents are bactericidal or merely bacteriostatic is not yet known. It may be that the inhibitory substances produced by lichens prevent temporarily the growth of some microorganisms and actually kill others.

MODES OF ACTION OF ANTIBIOTICS

Many theories have been offered to account for the phenomena of antibiosis. One of the oldest was the theory that more or less specific "excretory products" were produced by the stronger organism in order to destroy the weaker. Some believed that exhaustion of essential food materials by the stronger strain was the cause of antibiosis. Others attributed inhibition to unfavorable changes in pH of the medium (Fulton, 1937).

It is believed that the mode of action of antibiotic substances upon bacteria consists largely in interfering with cell multiplication. This may be accompanied by a marked effect upon certain essential metabolic processes. Bacteria subjected to the action of an antibiotic substance may develop mechanisms that render them resistant to the action of the substance (Waksman, 1944).

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culture in that substrate. These substances have been described as of a toxic nature (Porter, 1938).

Many organisms are capable of producing substances which are injurious to their own development; these are known as iso-antagonistic. Even more organisms are capable of producing substances which are injurious to other organisms growing close to them; these are known as hetero-antagonistic. This is the chief reason why certain molds and bacteria are capable of growing in practically pure cultures even in a non-sterile environment.

Examples have been presented directing attention to the retarding or the stimulating effect of the association of micro-organisms upon the development of cultures. It is a matter of common observation that the checking of vegetative development is usually a stimulus to spore production. This is seen many times when mycelial development is sharply inhibited by the presence of another organism (Porter, 1938).

Bacteria and molds affect their surroundings; in these effects may be discovered some of the explanations to account for staling and inhibitory phenomena.

Staling and inhibition are among the better known phenomena associated with competition among microorganisms. The term "staling substances" refers to the substrates that check the growth of the culture in the medium in which staling occurs,

or which renders that medium unfit for growth of other cultures. "Inhibitory substances" refers to substances that will check the growth of other organisms growing simultaneously on the same substrate (Porter, 1938).

It has been a frequent observation that acids are produced by microorganisms during the course of growth and development. An unfavorable hydrogen ion reaction often checks vegetive development and may inhibit spore germination. Acids produced during metabolism have therefore been suggested as the cause of both staling and inhibition (Porter, 1938).

Enzymes produced in cultures have been credited as possible causes of destruction of bactericidal agents of microorganisms as has been demonstrated in the case of the enzyme, penicillinase, which brings about the destruction of the antibiotic substance penicillin (Waksman, 1944).

Among the various types of antagonism, the most definite and the one which is best understood is that which results in the formation of antagonistic substances. This has been discussed previously in the case of molds and actinomycetes. The nature of these substances or toxins when produced by different bacteria and molds is not always the same. Some are destroyed by boiling, by exposure to light or filtration; others are resistant to heat and to ultraviolet rays; some are readily adsorbed by filters, from which they can be removed by special

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It has often been observed that certain organisms produce pigment in the presence of others and that these pigments are in some way associated with the phenomena of antagonism; for example, V. cholerae produced, in the presence of Sarcina lutea, a dark violet pigment, which is accompanied by an increase in agglutination and virulence (Schwentker and Complolier, 1939). Penicillium africanum produces a more intense pigment in contact with other fungi, such as A. niger; this pigment accumulates in the mycelium of the latter, which may thereby be killed (Doebelt, 1909).

The term "lysobacteria" has been applied to those microorganisms capable of dissolving living and dead organisms. Actinomycetes are known to have bacteriolytic properties as well as bacteriostatic and bactericidal activities (Welsch, 1942). Interest in the lytic agents is dominated by the possible use of bacterial lysates for vaccination purposes.

Bail (1925, 1929) suggested that there exists for every bacterium a typical constant number of living cells capable of living in a given space. When this concentration is reached, multiplication comes to a standstill without the nutrients being exhausted or toxic substances produced. The same is believed to hold true when two bacteria live together. If the limiting concentrations of the two organisms are different, the

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one with a higher concentration value will repress the other; the weaker species may check the stronger one when planted in a sufficient excess.

Fulton (1937) found that inhibition among bacteria may be weakened or entirely lost, depending upon the medium upon which the bacteria are grown.

The temperature surrounding the culture may be an influencing factor. Some consider the age of the culture likewise a factor in regulating the nature of the metabolic products. Reid (1935) believes that light is a factor.

Porter (1924, 1932) recognized that different organisms exhibit varying degrees of inhibition as well as different mechanisms of inhibition. The morphological effects produced by antagonists comprise changes in form, size and structure of hyphae, direction of growth as well as complete cessation of growth and abbreviation of hyphal segments.

The formation of an antibiotic substance is greatly influenced by the strain of the organism, the composition of the medium, and the condition of growth. We have seen that some antagonistic microorganisms produce more than one antibiotic substance. It is also definitely established that some substances or closely related compounds may be produced by more than one organism.

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PRACTICAL APPLICATIONS

Every plant and animal is subject to infection by a number of bacteria, fungi, and protozoa. The disease producing microorganisms find their way to the soil either in the excreta of the hosts or in their dead and infected remains. The fate of all the bacteria causing typhoid, dysentery, diphtheria, cholera, pneumonia, tuberculosis and numerous other diseases remained unknown for some time. The soil was searched for bacterial agents of infectious disease until the conclusion was reached that these do not survive long in the earth. It was suggested that the rapid destruction of these organisms is brought about by soil-inhibiting microbes antagonistic to the pathogens (Waksman and Woodruff, 1944).

The soil contains a number of different types of microorganisms antagonistic to various bacteria belonging to both the Gram-positive and Gran-negative groups. By enriching the soil with the specific bacteria, the corresponding antagonists increase and can be readily isolated. Escherichia coli was used for enrichment purposes (Waksman and Woodruff, 1940). Since this organism not only survived in sterile soil but actually multiplied there at a very rapid rate, it is known that the physical and chemical soil conditions had no injurious ef-

fect upon it. The addition of several hundred living cells of E. coli to a gram of sterile soil (260,000 per 100 gm of soil) resulted in an increase in ten days at 28° C. to 149 million per gram of soil. This tends to prove that when soil is freed from other organisms, it is a favorable medium for the multiplication of E. coli and that some of the soil organic matter is available to this organism as a source of energy, or it may have been available by the sterilization of the soil. However, the enrichment of fresh soil with large numbers of living E. coli cells led to their rapid disappearance. The destruction of E. coli was brought about by the development of certain antagonistic microbes which were able to multiple very quickly in the enriched soil. The total number of bacteria in the enriched soil increased greatly, because the cells of E. coli served as good nutrients for many of the soil microorganisms, especially the antagonists.

Investigations have been made of the effect of the activated sludge process as used in municipal sewage disposal plants on the removal or inactivation of a mouse adapted strain of poliomyelitis virus. Virus suspension 1:300 was used in sludge concentrations of 1,100, 2,200, and 3,300 p.p.m. with aeration of zero, six and nine hours. The results indicate that activated sludge in amounts as low as 1,100 p.p.m. with six hours aeration will remove or inactivate the virus to a sufficient extent to reduce greatly infectivity for mice injected

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Investigations have been made of the effect of the activated sludge process as used in municipal sewage disposal plants on the removal or inactivation of a more adapted strain of poliomyelitis virus. Virus suspension 1:500 was used in sludge concentrations of 1,000, 2,500, and 5,000 p.p.m. with aeration of zero, six and nine hours. The results indicate that activated sludge in amounts as low as 1,000 p.p.m. with six hours aeration will remove or inactivate the virus to a sufficient extent to reduce greatly infectivity for mice injected

intracerebrally. Heavier concentrations of sludge with longer aeration periods largely eliminate infectivity (Maxcy, 1940).

The survival of B. typhosus received particular attention in the study of mixed populations in sewage. This organism was found to survive for a much longer period of time in sterilized than in unsterilized tap water (Frost, 1904). The presence of certain bacteria, notably P. fluorescens, was shown to reduce considerably the survival period of the pathogen. Sewage was further shown to contain substances directly toxic to B. typhosus. It was also demonstrated that the destruction of this organism in sewage may be brought about by the numerous protozoa inhabiting this substrate.

Many attempts have been made to utilize microorganisms for the control of various diseases in plants, animals and man. Certain bacteria have been found to prevent the injurious action of the plant pathogen by direct repression as is shown for Helminthosporium on wheat (Porter, 1924). The reduction of potato scab by plowing under a green rye crop has been explained as due to the development of other organisms, such as saprophytic actinomycetes, which suppress the growth of the pathogenic Actinomyces scabies (Sanford, 1926). Various other experiments point to the possibility of suppressing the growth and infectiousness of plant pathogens by the activities of different soil microorganisms. Chudiakov (1935) isolated two bac-

teria which were capable of bringing about the lysis of different species of Fusarium and other fungi. These bacteria were widely distributed in the soil; they were absent, however, in certain flax-sick soils, in spite of the abundance of Fusarium. When this organism was introduced into soils containing the active bacteria, the fungus did not develop and plant disease did not occur.

A few antibiotics have been discovered which aid in controlling diseases of man and animals. Penicillin has had extensive chemotheropeutic application. The number of diseases and infections which are favorably influenced by penicillin therapy continues to increase as experience in the use of the drug is gained. It has proven to be effective against hemolytic streptococcus and pneumococcus infections, as well as various staphylococcal infections; it has been used to control local lesions of the eye, which are caused by Staphylococcus aureus. It is effective against sulfonamide-resistant strains of pneumococci (Waksman, 1944).

Fleming (1929) suggests that penicillin could be used as a dressing for septic wounds. This preparation has little toxic effect and seems to be superior to dressings containing active chemicals. As compared with sulphonamide drugs, it is not inhibited by tissue constituents and pus, thus offering a definite advantage from a chemotherapeutic point of view (Abraham and

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Chain, 1940).

Mice infected with a Type III pneumococcus can be protected by treatment with penicillin. Less penicillin and fewer injections are needed for protection when treatment is given at the same site as the infection. The number of active units of penicillin needed for protection is independent of the degree of purity of the preparations tested (McKee and Rake, 1942).

Recently, MaHoney, Arnold, and Harris (1943) made a report of penicillin treatment of early syphilis. The penicillin treatment consisted of an intramuscular injection of 25,000 units of the drug at 4-hour intervals, night and day, for eight days. The total number of injections was 48, and the total amount of the drug was 1,200,000 units. The gluteal muscle was the site of injection. Some mild but definite clinical manifestations were observed during the first eight hours of treatment. The patients complained of general malaise and mild headache. Temperature elevations not in excess of 2° F. were recorded. The regional lymph glands became enlarged and tender. The results of the blood studies indicate that the therapy was responsible for a more or less rapid and complete disappearance from the blood stream of the reacting substance which is usually associated with activity in early syphilis.

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It has been well established that penicillin is extremely effective in gonococcic infections. Even after infection has

been present for months, it can still be rapidly controlled (Bloomfield, Rantz and Kirby, 1944). Although penicillin is an effective chemotherapeutic agent in gonococcal infections, a few cases resist treatment. This evidence suggests that either penicillin-resistant strains of the gonococcus occur in nature or the organism acquires resistance to penicillin in vivo (Bahn, Ackerman and Carpentu, 1945).

Dawsen and Hobby (1944) reported observations made in one hundred cases which were treated with penicillin. This clinical study demonstrated that penicillin is a remarkably effective agent in the treatment of infections due to staphylococci, pneumococci, streptococci, gonococci, meningococci and diphtheria bacilli. A favorable response was obtained in 15 out of 18 cases of staphylococcic bacteremia. The results in 19 cases of staphylococcic infection without bacteremia were equally impressive. In 3 out of the 4 cases which failed to respond, the infecting organism was subsequently found to be resistant to penicillin in vitro. In chronic osteomyelitis the results were satisfactory only when penicillin therapy was used in conjunction with adequate surgery.

Herrell (1944) reported his observations on the clinical use of penicillin in 62 cases. Among the cases in which penicillin was used were infections due to Staphylococcus aureus, Neisseria gonorrhoeae, streptococci, actinomycetes, and micrococci. Satisfactory results were obtained in 48 of the 62 cases.

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Evans (1944) reported two cases of staphylococcic meningitis and 1 case of pneumoccic meningitis as having been cured with penicillin at Lawson General Hospital. Pencillin seems to offer more hope for cure of these maladies than any other substance known at the present time.

Pencillin has been shown to have no effect upon Mycobacterium tuberculosis. Other substances, however, such as streptomycin have been found to inhibit the growth of both M. tuberculosis and M. phlei (Schatz and Waksman, 1944). Streptomycin appears to be a promising antibiotic substance from the point of view of practical utilization against the human TB organism, because of its relatively greater in vitro activity against this strain of M. tuberculosis and its lower toxicity.

Gramicidin injected intraperitoneally into white mice was found by Dubos (1941) to exert a therapeutic action against experimental peritonitis caused by pneumococci and streptococci. However, it is almost completely ineffective when administered by the intravenous, intramuscular, or subcutaneous routes.

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I. V. Toroptsov and Filatova (1944) have reported the use of phytoncides, bactericides of plant origin, in the treatment of infected wounds. Although they began to administer phytoncide treatment during the summer when the concentration of phytoncides in onions is apt to be low, unusually gratifying results were obtained. Almost all of their patients had been pre-

pitalized for a long time with amputations of the extremities which had not healed. Failure of the tissues to regenerate was undoubtedly due to the purulent inflammation in the wounds. The regenerative processes became more marked after the first treatment with phytoncides. The daily dressings necessitated by the treatment did not give the wound the necessary rest, but regeneration proceeded more rapidly nevertheless. This they attributed to the powerful bactericidal effect of phytoncides.

SUMMARY AND CONCLUSIONS

Bacterial associations are of frequent occurrence. Organisms are rarely found growing as pure species in their natural habitat. The interrelationship may be favorable, in which case either one member or both members of the partnership are benefited; or the interrelationship may result in a harmful effect of one member on the other. This latter relationship, which is called antibiosis, is particularly important because of the part it plays in combating human and animal diseases. Antibiotics extracted from cultures of microorganisms are used in the treatment of infectious disease.

Many useful antibiotics have been discovered, as for example gramicidin, tyrothricin, streptothricin, streptomycin and the most important of all, penicillin.

The important organisms known at the present time to be capable of producing antibiotic substances are molds, yeasts, actinomycetes, spore-forming and non-spore-forming bacteria, viruses and lichens. The antibiotic substances produced by these organisms are primarily bacteriostatic in nature. Their bactericidal activities are of only secondary consideration.

One of the characteristic properties of antibiotic agents is their selective antibacterial action. Some affect Gram-pos-

itive bacteria chiefly and act only to a very limited extent upon Gram-negative organisms, while others have the capacity of inhibiting the growth of certain bacteria belonging to each of these groups.

To obtain an antibiotic substance, the antagonistic organism is grown on a culture medium, the composition of which, as well as the time and temperature of incubation, is determined by the greatest production of the active substance. Since these organisms are usually aerobic in nature, proper aeration of the culture is essential; hence shallow layers of liquid culture media for stationary cultures or forced aeration for submerged cultures are used. The formation of the antibiotic substance is greatly influenced by the strain of the organism, the composition of the medium and the conditions of growth.

Among the molds the members of the genera Penicillium and Aspergillus produce antibiotic substances which are particularly important. The mold Penicillium notatum excretes three important substances: (1) chrysogenin, a yellow pigment, (2) penicillin, (3) notatin. Penicillin has a strong antibacterial action. Gram-negative bacteria are least sensitive and pyogenic cocci most susceptible.

Aspergillus fumigatus also produces three antibiotic substances: (1) fumigatin, (2) fumigacin and (3) gliotoxin.

Aspergillus flavus produces two antibiotic substances,

aspergillic acid and flavicidin. The production of flavicidin instead of aspergillic acid seems to be a function both of the type of medium and the method of cultivation.

Flavicidin is similar to penicillin in several wqys: (1) both are very active against Gram-positive organisms, and only a little active against Gram-negative bacilli, (2) both protect mice in equal degree against pneumococcus infection, (3) both are highly soluble, (4) cultures resistant to the action of penicillin are resistant also to flavicidin but not to other antibiotic substances (5) an enzyme active against penicillin is active also against flavicidin, but not against other antibiotic substances.

Yeasts have been shown to contain substances toxic for themselves and for some bacteria and molds. In the case of both molds and bacteria the type of growth is altered.

Several antibiotic substances have been obtained from actinomycetes. Actinomycetes possessing antagonistic properties against bacteria and molds are widely distributed in nature, especially in soils and composts.

Among the spore-forming bacteria, the B. brevis, B. mesentericus and B. mycoides have proved to be most important. Pyocyaneus and fluorescens groups and members of the colon typhoid group are the most important representatives of antagonistic non-spore-forming organisms. B. brevis yields two

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crystalline polypeptides, gramicidin and tyrocidine, which are both endowed with antibacterial activity but differ in many other biological and chemical properties.

Antagonistic microorganisms which produce more than one antibiotic are rather common. It has also been established that some substances or closely related compounds may be produced by more than one organism.

The antagonistic effects of saprophytic soil microorganisms upon pathogenic bacteria and molds were at first visualized as resulting from competition for food between these two groups. It was soon recognized, however, that this was not a sufficient explanation for all observed phenomena, included under the term "antibiosis", and several other theories of antagonism were formulated. The type of antagonism best understood is that which results from the formation of specific antagonistic substances.

Antagonistic organisms cause the destruction of pathogenic bacteria in soil, water, sewage and within the animal body. The survival of disease-producing bacteria is checked in nature by antagonistic organisms which are abundant in soil and water.

Certain bacteria have been found to prevent the injurious action of a plant pathogen by direct repression. Saprophytic actinomycetes suppress the growth of pathogenic Actinomyces

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scabies thus reducing the potato scab.

Of the various antibiotic agents, penicillin is the one which has found great chemotherapeutic application. Penicillin has been found to be a remarkably effective agent in treatment of infections due to staphylococci, pneumococci, streptococci, gonococci, meningococci, diphtheria bacilli, Neisseria gonorrhoeae, actinomycetes, and micrococci.

Penicillin has been shown to have no effect upon Mycrobacterium tuberculosis. Streptomycin, however, has been found to inhibit the growth of both. M. tuberculosis and M. phlei. Streptomycin is a promising antibiotic substance from the point of view of practical utilization against the human TB organism.

Phytoncides, volatile substances produced from higher plants are bactericidal. Phytoncides have been used in the treatment of infected wounds.

If the antibiotics and their activities are properly controlled, it will give us much insight into the proper care of cultures, and a natural and effective method of controlling human, animal, and plant diseases.

ABSTRACT

Microbial associations of various types were reviewed: symbiosis, the living together of two or more organisms in friendly association, each receiving mutual benefit; commensalism, the living together of two species, one of which is benefited and the other neither benefited nor harmed; synergism, the association in which two bacteria growing together can form products which can be produced by neither growing alone; parasitism, the living or preying of organisms on the tissue of plants and animals; and antibiosis, the situation where there is a harmful effect of one roganism on the other.

In 1877, Pasteur discovered that anthrax in sensitive animals can be repressed by inoculating B. anthracis together with other bacteria, such as species of Streptococcus. Garré in 1887, Bouchard in 1889 and Freudenreich in 1888 found that some common saprophytes produce substances antagonistic to the colon-typhoid group of bacteria and also to many others. In 1904 Prescott and Baker observed that in a mixture of E. coli and S. faecalis, the former organism increased at a more rapid rate at the beginning; then the S. faecalis gained the ascendency and finally outgrew the E. coli present. In 1915 Twort observed a lytic action in a culture of staphylococcus. In

1916, D'Herelle observed the same phenomenon and named the lytic principle "bacteriophage". In 1920, Theobald Smith and D. E. Smith reported that B. paratyphosus B, after it had grown in lactose bouillon for four to six days, prevented the development of gas by B. coli when this was added. Greer and Nyhan, in 1928, prepared several water supplies and showed that one member of a pair almost always outgrew or destroyed the other member. In 1929, Fleming discovered penicillin. In 1939, Dubos discovered gramicidin. In 1940, Hoogerheide and McDonald isolated a substance from soil bacilli which inhibited encapsulation of Friedländer's bacillus (Klebsiella pneumoniae) and was also highly bactericidal for Gram-positive microorganisms. In 1941, Waksman and Woodruff isolated an Actinomyces from the soil which possessed strong bacteriostatic and bactericidal properties. In 1942, Dubos and Hotchkiss discovered tyrothricin and Waksman and Woodruff discovered streptothricin.

Organisms capable of producing antibiotic substances are classified into five groups: (1) molds, (2) yeasts, (3) actinomycetes, (4) bacteria, both spore-forming and non-spore-forming, (5) viruses, and (6) lichesn. A case of antibiotics in higher plants is also noted.

The mold, P. notatum, excretes three important substances: (1) chrysogenin, a yellow pigment, (2) penicillin, (3) notatin. Two methods of preparing penicillin were discussed. The cup assay method of standardizing penicillin was described. Peni-

cillin is effective against hemolytic streptococcus, pneumococcus, and various staphylococcus infections.

Three antibiotic substances, namely, fumigatin, fumigacin and gliotoxin, are produced from A. fumigatus. Of these three compounds, fumigacin offers the greatest promise as chemotherapeutic agent.

A. flavus produces aspergillic acid and flavicidin, a substance resembling penicillin in many ways. The production of flavicidin instead of aspergillic acid is a function of both the type of medium and the method of cultivation. A method of obtaining the active antibacterial substance is described.

Flavicidin and penicillin are similar in several respects. They are both very active against Gram-positive organisms, but only a little active against Gram-negative bacilli; both protect mice in equal degree against pneumococcus infection; both are highly soluble; cultures resistant to the action of penicillin are resistant also to flavicidin but not to other antibiotic substances; an enzyme active against penicillin is active also against flavicidin, but not against other antibiotic substances. Flavicidin, however, seems to be more toxic than penicillin.

Yeasts contain substances toxic for themselves and for some bacteria and molds. Rose yeasts contain a substance which is antagonistic to molds but not to yeasts. Yeasts were found

to contain a substance which inhibits the action of sulfanilamide against S. hemolyticus and other streptococci and pneumococci. In the case of both molds and bacteria the type of growth is altered.

Several antibiotic substances have been isolated from actinomycetes. They are highly selective and may be bacteriostatic, bactericidal or bacteriolytic.

Actinomycetes are widely distributed in nature, especially in soils and in composts. A method of demonstrating actinomycetes and other antagonistic microorganisms in the soil was described.

Some antibacterial properties of a species of Actinomyces belonging to the A. albus group were reviewed. This was shown to be a bacteriolytic organism. Bacteriolysis of living bacteria by this organism is two-step reaction: first, the susceptible cells are killed by the selectively bactericidal lipoid; second, those dead cells are then dissolved by the bacteriolytic enzyme.

The antibiotic substance, actinomycin, produced from the soil organism A. antibioticus was described. Another antagonistic organism which was found to be a strain of A. lavendulae was isolated. The antibacterial substance produced by this organism is streptothricin. Streptomycin is an antibiotic obtained from Actinomyces griseus.

Several spore-forming bacteria are known to have an inhibitory effect on other bacteria. Representatives of these are B. subtilis, B. mesentericus and B. brevis.

Three crystalline preparations, highly bactericidal for Gram-positive microorganism, have been obtained from the Gram-negative organism, B. brevis. A method of isolating them has been described. One of these, Gramicidin, is particularly important as an antibiotic.

Gramicidin and tyrocidine, both crystalline polypeptides yielded by cultures of B. brevis, were compared.

Other spore-forming bacteria, such as B. mesentericus, produce substances antagonistic not only to bacteria, but also to molds.

Of particular importance among the non-spore-forming bacteria possessing antagonistic properties are the pyocyaneus and fluorescens groups, and the members of the colon-typhoid group. P. aeruginosa, P. fluorescens and the colon bacillus have been discussed.

It is known that some microorganisms are capable of destroying viruses and that some viruses are capable of antagonizing other viruses. Examples of such occurrences have been given.

Antibiosis as exemplified in lichens was reviewed. It

has been found that Gram-positive bacteria including several pathogenic types are inhibited, but Gram-negative bacteria are generally not susceptible to the antibiotic substance of lichens.

The various theories offered to account for antibiosis were reviewed. Exhaustion of food nutrients, interference with cell multiplication are considered to be causes of inhibition. There are examples of retarding or stimulating effects of the association of microorganisms upon the development of cultures. Staling and inhibition are other well known phenomena associated with competition among microorganisms. The production of acids, enzymes, or pigments have been considered as possible causes of antagonism.

The medium upon which the bacteria are grown, the temperature surrounding the culture, and the age of the culture are considered to be influencing factors.

A few practical applications have been discussed. It is thought that the destruction of bacterial agents of infectious disease is brought about by soil-inhibiting microbes antagonistic to the pathogens. It has been shown that by enriching the soil with the specific bacteria the corresponding antagonists increase and can be isolated. Examples of treating sewage have also been described.

Various examples of prevention of injurious action of plant pathogens by bacteria have been mentioned.

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Various examples of prevention of injurious action of plant pathogens by bacteria have been mentioned.

The uses of microorganisms for the control of various diseases have been discussed. Penicillin is effective against hemolytic streptococcus, pneumococcus, and staphylococcal infections. It has also been used as a dressing for septic wounds and in the treatment of infections due to gonococci, menimgococci and diphtheria bacilli. It has also been effective in the treatment of infections due to Neisseria gonorrhoeae, actinomycetes and micrococci.

Streptomycin has been used in the treatment of tuberculosis.

Gramicidin has a therapeutic action against experimental peritonitis in white mice.

Phytoncides, bactericides of plant origin, have been used in the treatment of infected wounds.

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